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ARTICLE

Analysis of *FMR1* $(CGG)_n$ alleles and FRAXA microsatellite haplotypes in the population of Greenland: implications for the population of the New World from Asia

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The fragile X syndrome is caused by the expansion of a polymorphic $(CGG)_n$ tract in the promoter region of the *FMR1* gene. Apparently the incidence of fragile X syndrome is rare in the population of Greenland. In order to examine population-related factors involved in stability of the $(CGG)_n$ sequence, DNA samples obtained randomly from the Greenlandic population were analysed for size and AGG interspersion pattern of the *FMR1* $(CGG)_n$ region and associated DXS548-FRAXAC1 haplotypes. In addition a large Greenland family with unstable transmission in the premutation range was analysed. The $(CGG)_n$ allele sizes in the Greenland population showed a narrow distribution similar to that reported for Asian populations. DNA sequencing of alleles with 36 CGG repeats revealed an $AGG(CGG)_6$ insertion previously reported exclusively in Asian populations and a high frequency of alleles with a $(CGG)_{10}AGG(CGG)_9AGG(CGG)_9$ or $(CGG)_9AGG(CGG)_9AGG(CGG)_6AGG(CGG)_9$ sequence pattern was found. Thus the data confirm the Asian origin of the Greenlandic (Eskimo) population and indicates that some $(CGG)_n$ alleles have remained stable for 15–30,000 years, since the population of the New World arrived from Asia via the Bering Strait.

Keywords: fragile X syndrome; *FRAXA*; *FMR1* gene; $(CGG)_n$ trinculeotide repeats; PCR; Eskimo

Introduction

Fragile X syndrome is the most common form of inherited mental retardation, with an incidence of 1 in 4–5000 males.[1,2] The condition is usually caused by expansion of an unstable $(CGG)_n$ trinucleotide repeat sequence in the promoter region in the *FMR1* gene (Xq27.3), leading to hypermethylation of a neighbouring CpG island and repression of *FMR1* mRNA transcription.[3–5] In normal individuals, the region is stably transmitted but polymorphic, with CGG repeats ranging in number from 5 to 52. In carriers of the unstable premutation, repeat lengths of 50–200 CGG are usually found and in affected individuals a full mutation of >200 repeats can be found.[6] Little is known about the factors involved in stabilising the

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$(CGG)_n$ tract in the normal population. Analyses of microsatellites located around the *FMR1* gene have demonstrated linkage for haplotypes associated with the disease.[7] Sequence analysis of $(CGG)_n$ alleles have shown that a large proportion of normal alleles are interrupted by an AGG triplet for each 9–10 CGG. However, analysis of normal alleles associated with haplotypes overrepresented among fragile X patients, led to the hypothesis that alleles with a tract of > 24 pure CGG repeats are predisposed to repeat expansion.[8] Several other studies have confirmed that long stretches of pure CGG repeats are associated with instability of the *FMR1* $(CGG)_n$ repeat.[9–12]

Studies of $(CGG)_n$ repeat structures in selected human populations have shown a high degree of conservation of the canonical $(CGG)_9AGG$ interruption pattern in different populations and have confirmed the proposed stabilising effect of AGG interruptions.[13,14] We have studied *FMR1* $(CGG)_n$ sequence patterns and DXS548-FRAXAC1 haplotypes in the population of Greenland in order to examine molecular factors influencing the stability of the human *FMR1* gene. We also present a large Greenland family segregating an unstable premutation in combination with a rare FRAXA microsatellite haplotype.

Materials and Methods

DNA Sample Material

Blood spots on filter paper, used for newborn screening of the Danish (Caucasian) and Greenlandic (Eskimo) population, were used as sample material. The population of Greenland originates from the Eskimo-Aleut population, which is believed to have migrated to North America ('The New World') from North-East Asia across a land bridge, formed at the Bering strait, in the late Paleolithic period 15–30,000 years ago.[15] The present population of Greenland is approximately 56,000, with a newborn population of 1,100. The uptake of the PKU screening is approximately 80%. The study was totally anonymous and the samples were taken randomly from a pool of daily routine samples. DNA was extracted from 3 mm filter paper discs as described.[16] The project is in agreement with the Helsinki declaration II, and has been approved by the local Science Ethics Committee, J No (KF) 01-182/96.

PCR Amplification of FRAXA Microsatellites

PCR amplification of *FMR1* $(CGG)_n$ repeats was performed essentially as described[16] using fluorescently labelled primers. DXS548 and FRAXAC1 microsatellites were amplified by multiplex PCR using primers labelled with FAM or TET fluorescent dyes (Perkin Elmer, Foster City, CA). Published primer sequences were used.[5,17] Thermal cycling was performed in a PTC200 'DNA engine' (MJ Research, Waltham, MA), with the temperature profile: 95°C for 2 min followed by 31 cycles of 95°C for 20 s, 62°C for 30 s, 55°C for 30 s and 72°C for 1 min, ending with a 7 min extension at 72°C.

DNA Sequencing

Purified PCR products were sequenced with a *Taq*FS Dye-Deoxy™ Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA) according to the manufacturer's instructions, using the internal sequencing primers 5'CGTGGTTTCAGTGTTTAC 3' (forward) and 5' CTCCTCCATCTTCTCTTC 3' (reverse). All sequences were analysed using an ABI373 DNA sequencer (Perkin Elmer, Foster City, CA).

Greenland Family with Unstable CGG Repeat: Ascertainment and Investigation

From our clinical diagnostic service we have identified one large family from Greenland with an unstable CGG repeat at the FRAXA locus, which segregated in four generations in 16 individuals, apparently without expanding to a full mutation.

The family was ascertained through IV-2 and IV-3. IV-2, the oldest of the two index brothers, is 6 years old. He is mildly psychomotorically delayed with no dysmorphic features or physical abnormalities. His language development has been delayed as well as his social skills. His mood is stable. IV-3, 5 years old, is the more severely affected of the two brothers, as he shows clear autistic features with hyperactive behaviour, perseveration, no expressive language and severe mood changes with temper tantrums. His general development is delayed. He has no dysmorphic features or physical abnormalities.

The repeat size was determined according to ref. 6. Interspersion analysis in premutation male carriers was done according to ref. 12. Haplotype analysis with PCR amplification of the microsatellites DXS548, FRAXAC1 and FRAXAC2 was done both as described under 'PCR amplification of FRAXA microsatellites' for two of the microsatellites, and with a radioactive method as described elsewhere.[18] Western blot analysis was performed as described elsewhere.[18] Analysis for FRAXE expansion was performed with standard Southern blot technique using the OxE18 probe on *Eco*RI blots and *Hind*III blots.[19]

Results

Distribution of $(CGG)_n$ Alleles and DXS548-FRAXAC1 Haplotypes in the Greenland Population

PCR analysis of *FMR1* $(CGG)_n$ alleles in DNA extracted from 101 newborn males sampled randomly from the Greenland population showed a distribution with two major peaks around 30 CGG (53% with 29–31 CGG) and 36 CGG (26% with 35–37 CGG). Only 8% of the alleles had fewer than 26 repeats (Figure 1). This is very similar to distributions reported for Asian populations.[20–22]

Comparison of $(CGG)_n$ allele sizes and linked DXS548-FRAXAC1 haplotypes showed that 85% of alleles with 29–31 CGG were linked to haplotype 7-3

and 88% of alleles with 35–37 CGG were linked to haplotype 6-4 (Figure 1).

Sequencing of FMR1 *(CGG)$_n$ in Greenlandic Males*

The $(CGG)_n$ sequence was determined for 85 DNA samples extracted from Greenlandic males by direct sequencing of PCR products. Twenty-three male alleles in the range of 19–44 CGG in repeat size were not sequenced due to shortage of sample material. The sequenced alleles are classified with respect to DXS548-FRAXAC1 haplotypes in Figure 2.

The sequenced alleles are generally highly interrupted by AGG triplets, with 92% of the alleles having two or three AGG interruptions. Most of the $(CGG)_n$ alleles analysed, had a sequence organisation of $(CGG)_{10}AGG(CGG)_9AGG(CGG)_9$ (Figure 2). The DNA sequencing also revealed a $(CGG)_6AGG$ insertion in 20% (17/85) of the samples (Figure 2). This insertion has previously been described exclusively in the Asian population,[20,23] thus our data support the theory of an Asian origin of the Eskimo component of the Greenland population.[15]

Greenlandic family: Haplotype Analysis, AGG Interspersion Analysis and Segregation of Premutation

Blood samples from 18 family members were investigated for the fragile X syndrome by Southern blot and PCR analyses. Sixteen samples showed expansion of the CGG repeat ranging in size from 57 to 70 (Figure 3a and b).

The premutation was shown to originate in I-1, who fathered eight obligate carrier daughters. In four of these, the repeat expanded by one or a few repeats; in two there was a stable transmission, and in two a regression by one or two repeats. In the following generation a further slight expansion occurred in those investigated, and in the youngest generation (IV) the two brothers showed increases in repeats to 70 and 67 CGG (Figure 3a and b).

To exclude possible mosaicism in the two index brothers we investigated skin biopsies from both and found expansions to be of the same size without evidence of a full mutation. Western blot analysis on protein extracts from skin fibroblasts likewise indicated the presence of FMRP protein (data not shown). Point

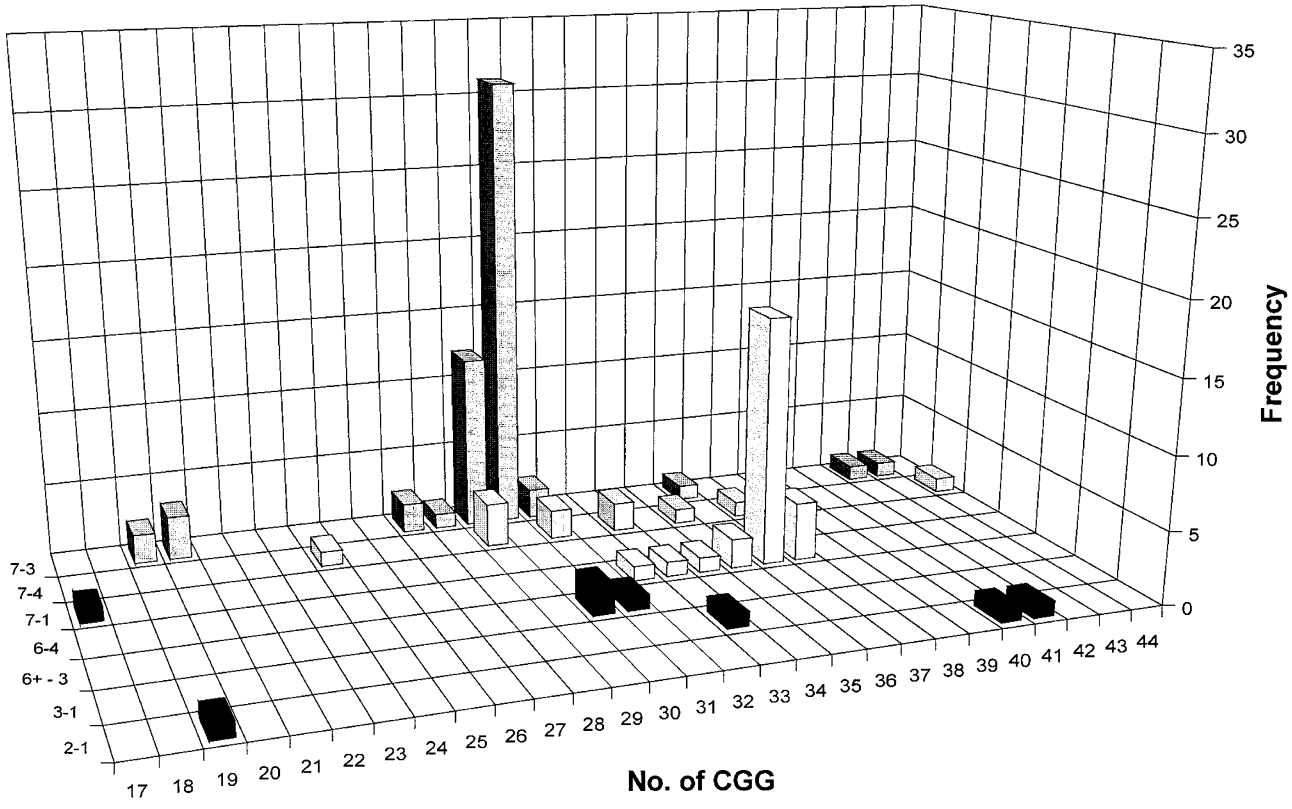

Figure 1 *Distribution of* FMR1 *(CGG)$_n$ repeats and DXS548-FRAXAC1 haplotypes in the Greenland population. The nomenclature of the haplotypes is according to Macpherson et al.*[34]

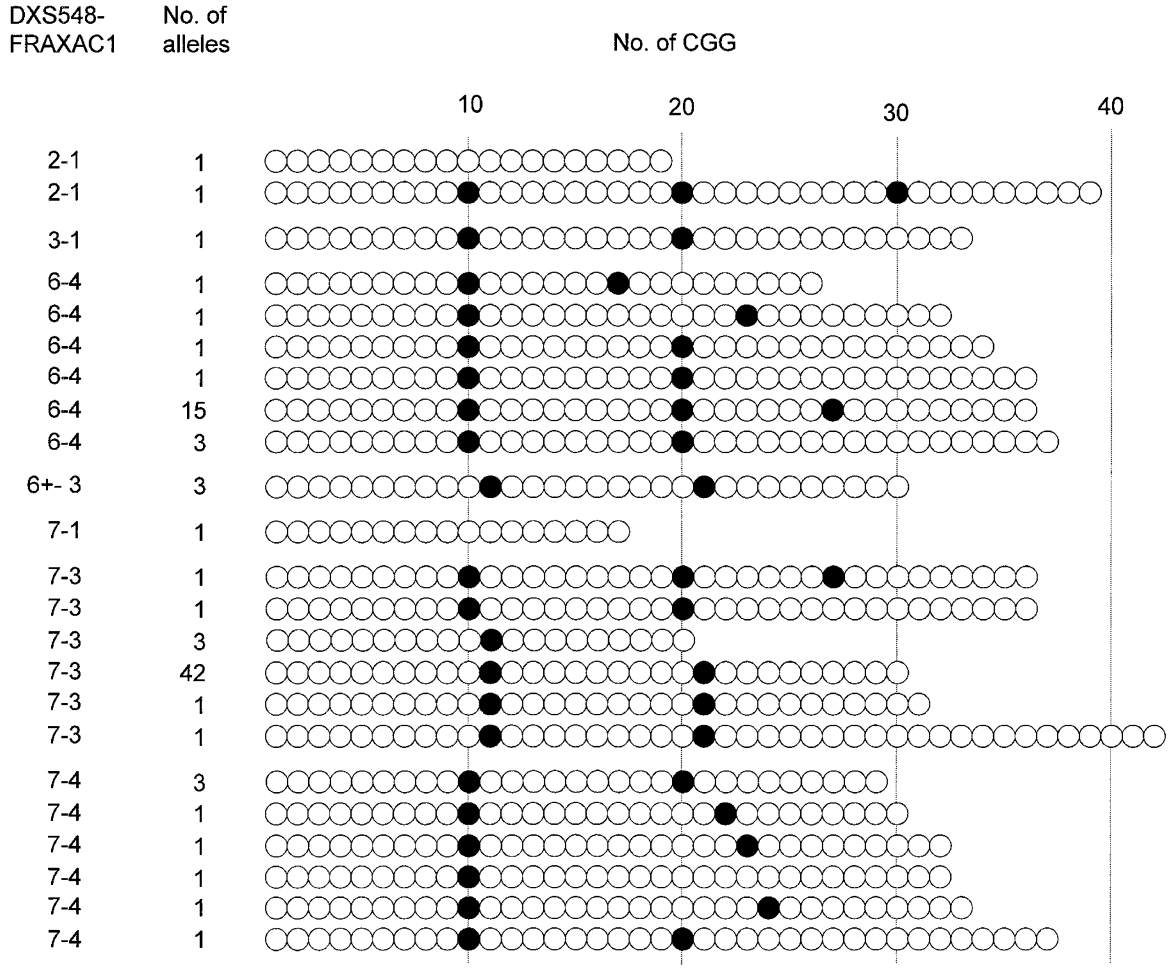

Figure 2 *AGG interspersion pattern of 85 sequenced* FMR1 *$(CGG)_n$ alleles from Greenland males. The alleles are classified according to the associated DXS548-FRAXAC1 haplotype (first column). Only one of each allele type is shown, and the number of each allele type is indicated in the second column. The AGG interspersion patterns are shown from the 5' to the 3' end. Open circles represent CGG triplets; solid circles represent AGG triplets. The number of triplets are indicated on top*

mutations in *FMR1* was ruled out in IV-2 by SSCP analysis[24] and FRAXE was ruled out by Southern blotting.

AGG interspersion analysis was carried out in four premutated males. All four samples showed a sequence pattern of $(CGG)_9AGG(CGG)_n$, with *n* ranging from 49 to 57 CGG (data not shown). Haplotype analysis showed the premutation carriers to possess the haplotype 2-4-6^+ for markers DXS548, FRAXAC1 and FRAXAC2.

Discussion

The distribution of *FMR1* $(CGG)_n$ alleles is considerably different in Caucasian and Asian populations.[6,20–22] Analysis of the CGG distribution in the Greenland population (Figure 1) showed similarities to the distribution in Asian populations, with a high frequency of 35–37 CGG and a lower frequency of alleles ranging from 20–23 CGG in size, compared with Caucasian populations. Although similarities to Asian distributions were significant, minor deviations were observed. The major difference is that the modal repeat length of Asian populations is 28–29 CGG repeats, whilst 30 CGG repeats is the modal repeat length in the Greenlandic population (Figure 1). Sequencing of the $(CGG)_n$ alleles and analysis of associated DXS548-FRAXAC1 haplotypes showed that the most frequent occuring $(CGG)_n$ allele in the Greenlandic population has a sequence organisation of $(CGG)_{10}AGG(CGG)_9AGG(CGG)_9$, and is associated with haplotype 7-3 (Figure 2). These alleles are typically Caucasian, whilst Asian modal alleles have the sequence organisation of

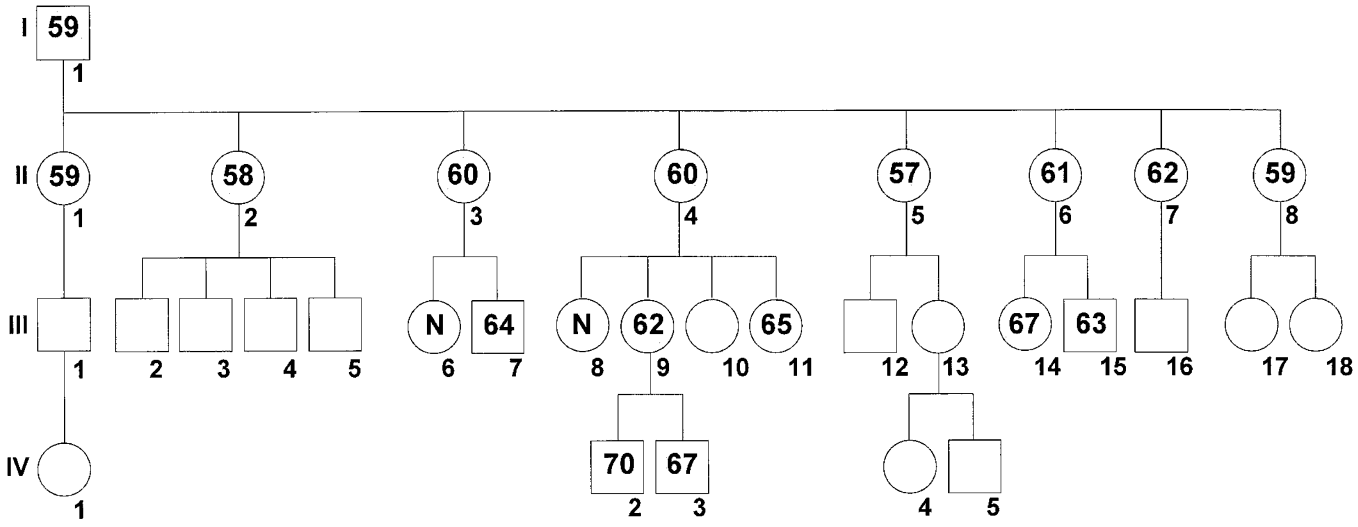

Figure 3a *Pedigree of a Greenland family. Shaded symbols refer to premutated individuals, the numbers inside the symbols refer to repeat number. All premutated individuals except III-14 were analysed in the same experiment to make size determination more accurate (see Figure 3b). N refers to a normal individual. Empty symbols refer to subjects not investigated*

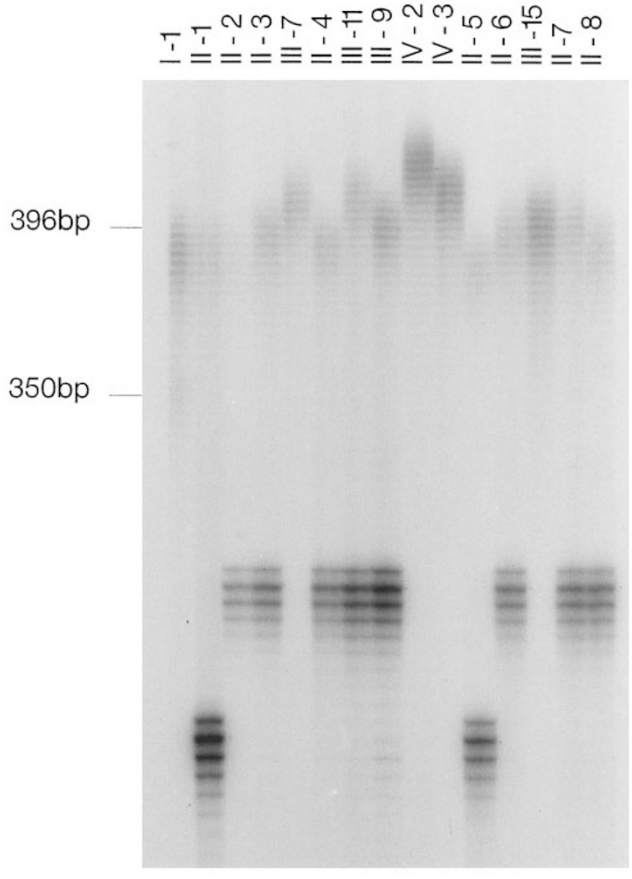

Figure 3b *PCR amplification of CGG repeat in the Greenland family, showing the instability of the repeat*

$(CGG)_9AGG(CGG)_9AGG(CGG)_9$ and are normally associated with haplotype 7-4.[20,22]

Alleles with 36 CGG repeats in the Greenlandic population were mainly associated with haplotype 6-4 (Figure 2). This is also different from Asian populations in which these alleles were found to associate with haplotype 7-4.[20,22]

However, both $(CGG)_{29}$ alleles associated with haplotype 7-3 and $(CGG)_{36}$ alleles associated with haplotype 6-4 exist in Asian populations;[14,22] thus the changed frequency of these haplotypes from the Asian to the Greenland population may reflect random genetic drift due to the relative small population sizes believed to exist in the late Paleolithic era.[15] It may also be caused by genetic mixture with the Danish population, which has been in contact with the Eskimo population since 1721.[25] Studies dealing with the frequency of Caucasian genes in the Greenland gene pool indicate that this frequency may be as high as 25% in most parts of the population.[26,27] However, a more systematic study of this problem is certainly needed.

Furthermore, the uptake of newborn screening in Greenland is only 80%. This may partly be due to a particularly low uptake in isolated communities. The genetic mixture of Eskimo and Caucasian genes is presumably low in such isolated communities, thus our sample material may be slightly biased towards samples with Caucasian mixture.

The existence of a $(CGG)_6AGG$ insertion, previously described exclusively in Asian populations,[20,23] in a large proportion of the Greenland $(CGG)_n$ alleles

studied here, confirms an Asian origin of the Greenlandic population, and adds new evidence for the 'out of Asia' theory of the colonisation of the New World 15–30,000 years ago.[15] The $(CGG)_n$ repeats in native Americans have been studied by several groups,[13,14,20] but none of these studies have found the $(CGG)_6AGG$ insertion in native Americans. This may be due to the relatively small sample sizes in these studies, but may also be caused either by a later migration of the Eskimo population compared with the Amerind and the Na-dene populations, as proposed in the 'three migrations-theory'[28] or by genetic bottlenecks during the population of the New World.[29]

DNA sequencing of the *FMR1* $(CGG)_n$ alleles showed a high proportion of AGG interruptions in the CGG repeat structure, with 92% of the alleles having two or more AGG interruptions (Figure 2). A high proportion of the alleles had the sequence organisation $(CGG)_{10}AGG(CGG)_9AGG(CGG)_9$ (49%) and $(CGG)_9AGG(CGG)_9AGG(CGG)_6AGG(CGG)_9$ (18%). These sequence organisations have been found in most 30 CGG and 36 CGG alleles analysed worldwide and in Asia, respectively, indicating that these alleles have remained stable since they were separated from the rest of the world by the Bering Strait dispersal 15,000 years ago. Thus, the data are in agreement with the hypothesis, that AGG interruptions have a stabilising effect on $(CGG)_n$ alleles. The longest pure CGG block among the normal Greenlandic alleles was 21 CGG repeats (Figure 2). Analysis of *FMR1* alleles in both Caucasian and Asian populations has led to the hypothesis that a proportion of the normal alleles may be unstable, predisposed to mutate to the fragile X full mutation.[8,30] In Caucasian populations these predisposed alleles would have long blocks of uninterrupted (> 24) repeats.[8] The absence of alleles with > 24 pure CGGs (Figure 2) in the Greenland population could indicate that most of the predisposed alleles may have been absent from the pool of founder chromosomes or were lost by genetic drift. It is interesting to note that the haplotype 6-4 (DXS548-FRAXAC1), occurs at a relatively high frequency in this population (25%). This is known in Caucasian populations to be the most frequent haplotype in the fragile X patient population. It is noteworthy that in the Greenlandic population, the 6-4 haplotype was mostly found in association with the $(CGG)_9AGG(CGG)_9AGG(CGG)_6AGG(CGG)_9$ allele, which is also found in Asian populations. However, comparing the frequency of fragile X syndrome in Asian and Caucasian population did not reveal differences,[31] although the 6-4 haplotype is twice as frequent in Asian populations. Furthermore, AGG interspersion analysis has shown the haplotype 6-4 in the normal Caucasian population to be associated with a different CGG/AGG pattern, containing an intermediate block of 11 to 12 CGG repeats.[10] This indicates, that risk haplotypes are not the same in different populations, and extrapolations cannot be made.

Data from the family presented show that an unstable premutated CGG repeat does occur in the Greenlandic population, although so far no full mutation has been demonstrated. However, only few patients have been investigated so far. The ascertainment of the family occurred through two young boys with deviant behaviour and development. We assume that the association of the abnormal phenotype with the premutation could be fortuitous, although there have been indications in the literature of a potential phenotypic effect of a premutation, as evidenced by premature ovarian failure in females[32,33] and an overrepresentation of intermediate alleles (41–60 CGG) in mentally retarded male populations.[1]

It is interesting to note that, although there is an overall tendency towards expansion, no expansion to full mutation occurred in the seven maternal transmissions studied in this family. These results confirm both that pure repeat lengths above 34 CGG may be unstable[12] and that premutations of < 70 CGG repeats in size seldom expand to full mutations.[6]

The haplotype (DXS548-FRAXAC1 2-4) segregating with this repeat was not otherwise found in the Greenlandic population sample investigated, but has been found in the Danish (unpublished observations) as well as in other Caucasian populations. It appears to be a rare 'risk' haplotype for the fragile X syndrome in the Danish population.

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